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PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT  
OF THE EYE

*mail*

**FIELD OF THE INVENTION**

The present invention concerns pharmaceutical compositions for the treatment of the eyes and more specifically for the treatment of disorders of the anterior segment of the eye.

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**BACKGROUND OF THE INVENTION**

The protective structures of the anterior surface of the eye include the eyelids, conjunctiva, and the cornea. The posterior surfaces of the lids are covered with a mucous membrane and the palpebral conjunctiva which reflects onto the eye to become the bulbar conjunctiva. The bulbar conjunctival epithelium is continuous with the corneal epithelium which accounts for about 10% of the anterior surface of the eye and is where most of the stationary refraction occurs.

The corneal epithelium is 4-5 cells thick and the superficial cells contain many microvilli. These aid in maintaining the moisture of the epithelial surface by promoting the adhesion of the tear film to the surface. This film lubricates the anterior surface of the eye to decrease the frictional forces arising from the persistent blinking movements of the eyelids, foreign particles on the surface of the eye, and the rotational movements of the eyeball. The tear film also transfers oxygen from ambient air to the cornea.

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The anterior surface of the eye is vulnerable to damages inflicted by various causes including mechanical abrasion of the cornea; contact lens wearing; spontaneous peeling of the epithelium; damaged epithelium and stroma following photo-refractive keratectomy; chemical burns; over exposure to ultraviolet light including sunlight; systemic diseases such as Sjogren syndrome, Steven-Johnson syndrome, Cicatricial pemphigoid syndrome; chronic edema of cornea with recurrent erosion of epithelium; impaired tear film formation, and conditions following damage of epithelia due to radial keratotomy.

Aging often causes disorders resulting from slow regeneration of the epithelium. The impaired regeneration and abnormality of the cells causes thinning of the epithelial layer and its impaired adherence to the basal lamina thus decreasing the ability of the cornea to retain the tear film leading to further epithelial damage.

Following injury to the corneal epithelium, nearby cells retract slightly, round up and begin an ameboid migration from the basal layer across the exposed basement membrane to cover the defect with a new monolayer of cells. These cells then take on the characteristics of a new basal layer and undergo mitosis to gradually fill in the defect with the full complement of four to five layers of cells. Present treatment for corneal wounds involves applying eye drops to the surface in order to protect the delicate healing process from erosion due to blinking and the other sources of friction. There are no currently used medicaments that promote the healing process itself. Attempts to administer fibronectin in order to promote healing of persistent defects of the corneal epithelium failed (Fukuda *et al.*, *Am J. Ophthalmol.*, 119(3):281-287, (1995)).

It would have been highly desirable to provide an ophthalmic composition capable of protecting the corneal epithelium and enhancing its healing and regeneration.

The rate of cell proliferation in many cell types has been correlated with the rate of cholesterol synthesis, and more specifically with the biosynthesis

of various intermediates in the cholesterol biosynthesis pathway and their by-products such as farnesylated proteins and others. Thus, inhibition of an early enzyme in the biosynthesis of cholesterol inhibits cell growth in cultured fibroblasts (McGuire *et al.*, *J. Biol. Chem.*, 268:22227-22230, (1993)). Factors  
5 which cause cholesterol efflux from cells (e.g. high density lipoproteins, HDL) alleviate the negative feedback inhibition of cholesterol synthesis and enhance growth of MDCK cells *in vitro* (Gospodarowicz *et al.*, *J. Cell. Physiol.*, 117:76-90, (1983)).

The cornea is an avascular organ obtaining nutrition from the  
10 vasculature of the limbus by diffusion. At the outer surface of the cornea the epithelium is essentially isolated from the plasma's large complexes such as HDL which hardly diffuse through the cornea. Thus, HDL which performs the "reverse cholesterol transport" from peripheral organs to the liver (Glomset, J.A., *J. Lipid Res.*, 9:155-167, 1968) is unable to perform this task in the corneal  
15 epithelium.

## SUMMARY OF THE INVENTION

The present invention is based on the surprising finding that high density lipoprotein (HDL), or a combination of its non-cholesterol lipid  
20 constituents (phospholipids, and other lipids such as triglycerides and glycerol), which are capable of forming reconstituted HDL, promotes normal healing and regeneration of damaged eye epithelium.

Both HDL and said lipid constituents were able to initiate the process of healing, to increase its rate, and to promote reversion of the damaged  
25 epithelium of the eye to the normal state, i.e. where the damaged area is covered again by layers of epithelial cells.

Thus, the present invention concerns a pharmaceutical composition for the treatment of disorders of the anterior segment of the eye comprising, as an

active ingredient, at least one agent capable of causing a net efflux of cholesterol from cells, together with an ophthalmologically acceptable carrier.

The term "*treatment*" refers to curing of the disorder of the eye, to alleviation of some of the undesired symptoms of various eye disorders, and/or to prevention of various eye disorders before they are manifested.

The term "*anterior segment of the eye*" refers to the corneal and conjunctival epithelium and includes the epithelial cells, as well as the glands present in the epithelium.

The term "*disorders of the anterior segment of the eye*" refers to disorders which cause physical damage to the corneal or conjunctival epithelium, to disorders which decrease the rate of regeneration of cells making up this epithelium, or to disorders causing diminished secretions from glands present in the conjunctival epithelium, or to a combination of some of these disorders.

Typical disorders of the anterior segment of the eye caused by physical or chemical damage are: mechanical abrasion of the cornea, corneal epithelial defects created by wearing contact lenses, corneal epithelial defects created by spontaneous peeling of the epithelium, corneal damage following photo-reactive keratectomy, injuries caused by chemical substances, damage caused by exposure to ultraviolet light, systemic diseases creating damage to the corneal epithelium and conjunctiva, for example, Sjorgren-Syndrome, Steven-Johnson Syndrome, Cicatricial Pemphigoid Syndrome, chronic edema of the cornea with recurrent erosion of epithelium and the like.

Typical disorders of the anterior segment of the eye caused by a decrease in the rate of generation of cells include deterioration of the eye due to old age or an anti-proliferative treatment.

AMENDED SHEET  
IPEA/EP

The pharmaceutical composition of the present invention may be administered to persons suffering from disorders which cause damage to the corneal or conjunctival epithelia, or in conjunction with treatments which are known to cause such damage, for example, laser or radial keratectomy or administration of various systemic or topical medications.

The active agents of the invention are those capable of causing a net efflux of cholesterol from cells. Locating candidate agents capable of generating a net cholesterol efflux, may be carried out, for example, by determining the net efflux of labeled cholesterol from cells according to the method described in Naphtali Savion and Shlomo Kotev-Emeth, *Eur. J. Biochem.*, 183:363-370 (1989). Briefly, confluent endothelial or smooth muscle cultures are allowed to incorporate  $^3\text{H}$ -cholesterol. The candidate to be tested as an effector of cholesterol efflux is then added to the cell culture and the percentage of radioactivity remaining in the cells after 24 hrs. is determined. Candidates which are able to significantly lower the amount of labeled cholesterol in these cells, are those which are capable of serving as active agents in the pharmaceutical compositions of the invention.

Preferably, the present invention concerns a pharmaceutical composition for the treatment of disorders of the anterior segment of the eye comprising as an active agent at least one compound selected from the group consisting of:

- i. high density lipoprotein (HDL);
- ii. a composition of matter termed "reconstituted HDL" and sphingolipids comprising phospholipids and at least one other lipid component of HDL other than cholesterol and cholesteryl-ester; and

iii. at least one HDL Apolipoprotein.

The term "*high density lipoprotein*" refers to lipoproteins which may be isolated from humans or other mammalian sources (e.g. bovine plasma), for example, as specified in Denis Gospodarowicz "*Methods for Preparation of Media, Supplements, and Substrata for Serum-Free Animal Cell Culture*", pp. 69-86, 1984, Alan R. Liss, Inc., New York, New York or other isolation methods based on the density of the HDL.

The term "*phospholipids*", refers to phospholipids which naturally occur in HDL such as phosphatidylcholine, phosphatidylserine and phosphatidylinositol. An example of "*sphingolipids*" is sphingomyelin.

The term "*and at least one other lipid component of HDL other than cholesterol and cholesteryl-ester*" refers to glycerides, glycerol and triglycerides. In accordance with the invention glycerides and triglycerides which are not present naturally in HDL, but have an analogous function to glycerides and triglycerides present in HDL may also be used. The composition of matter comprising the non-cholesterol and the non-cholesteryl-ester lipid components of HDL (generally phospholipids, triglycerides and glycerides) is termed "*reconstituted HDL*" (Gillote *et al.*, *J. Biol. Chem.*, 271:23792-23798, 1996). This term refers to a complex comprising phospholipids, triglycerides and glycerides, which differs from natural HDL by the absence of cholesterol, cholesteryl-esters, and apolipoproteins.

Reconstituted HDL particles are prepared by the chelate dispersion/Bio-Bead removal technique (Sparks *et al.*, *J. Biol. Chem.* 267:25830-25838, 1992). Typically, compounds which are used in intravenous nutrition as a source of essential fatty acids, are suitable for serving as the lipid components of HDL. Example being Intralipid™, (Pharmacia AB, Sweden), Lipofundin™ (Braun Melsungen AG, Germany) and others.

The term "*HDL apolipoproteins*" refers typically to apolipoprotein A-I, A-IV and E-apolipo-proteins or a combination thereof, either isolated from a human or mammalian source (Savion and Gamliel, *Arteriosclerosis*, 8:178-186, 1988). Apolipoprotein-E is purified according to Wernette-Hammond *et al.*, *J. Biol. Chem.*, 264:9094-9101, 1989. The HDL apolipoproteins may also be prepared by various genetic engineering methods described in Breslow, *et al.*, *Proc. Natl. Acad. Sci.*, 79:6861-6865, 1982. For example: Human Apolipoprotein A-I gene can be prepared according to the method of Karathanasis *et al.*, *Proc. Natl. Acad. Sci.*, 80:6147-6151, 1983; Human Apolipoprotein A-IV gene according to Elshourbagy, *et al.*, *J. Biol. Chem.*, 262:7973-7981, 1987; and Human Apolipoprotein E gene according to Das, *et al.*, *J. Biol. Chem.*, 260: 6240-6247, 1985; Paik, *et al.*, *Proc. Natl. Acad. Sci.*, 82:3445-3449, 1985.

The composition of the present invention may further comprise albumin.

Albumin is the most abundant plasma protein and serves as the plasma carrier of free fatty acids. Each albumin molecule has 27 binding sites for fatty acids. Albumin may thus serve as a scavenger for toxic free fatty acids released by damaged anterior chamber tissue included in reconstituted HDL.

The pharmaceutical compositions of the invention may further comprise other ingredients having ophthalmic effects, especially those which are known to facilitate healing and regeneration of cornea and conjunctiva such as growth factors, for example, keratinocyte growth factor (KGF/FGF7), or

epidermal growth factor (EGF) and other growth factors of the EGF family known in the art; various attachment factors such as laminin or fibronectin, and extracellular matrix components such as collagen, heparan sulfate proteoglycans and others.

5           The pharmaceutical compositions of the invention may also include agents capable of providing ultraviolet light protection, such as oxybenzone 3%, and other such preparations known in the art.

10           The pharmaceutical compositions of the invention should be administered in the form of eye drops or eye salves together with ophthalmically acceptable carriers. The composition may be in the form of an emulsion, micelles liposomes, etc. The concentration of the active ingredients in the composition should be in the range of 0.1-20%, preferably 0.2-10%, most preferably 0.2-2%.

15           Some disorders of the eye that are to be treated by the pharmaceutical compositions of the invention include diminished liquid clearance from the eye causing water retention which eventually leads to the rupture of the eye membranes. In such cases, it is preferable that the compositions of the invention be presented in a hyperosmotic formulation which can serve to draw excess liquid from the eye. Such hyperosmotic formulation may be formed, for  
20           example, by the addition of NaCl to the composition.

By another aspect, the invention comprises a method for the treatment of disorders of the anterior segment of the eye comprising administering to a subject in need of such treatment at least one agent capable of causing a net efflux of cholesterol from cells.

25           By another aspect, the invention comprises use of at least one agent capable of causing a net efflux of cholesterol from cells for the preparation of a medicament for the treatment of disorders of the anterior segment of the eye.

By yet another aspect, the present invention concerns a storage preparation for storing and maintaining isolated corneas, for example, in an eye



bank. In order to maintain the viability of epithelial cells as well as the exposed endothelium of the eye, it is preferable to add to the storage medium an effective amount of at least one agent capable of causing a net efflux of cholesterol from cells, as explained above.

5           The invention now will be illustrated with reference to some non-limiting drawings and examples.

### BRIEF DESCRIPTION OF THE DRAWINGS

10           Fig. 1 shows fluorescein staining of untreated damaged eye cornea 5 days after surgery (x 16);

          Fig. 2 shows fluorescein staining of untreated damaged eye cornea 12 days after surgery (x 16);

          Fig. 3A shows the histological appearance of normal cornea;

15           Fig. 3B shows the histological appearance of untreated damaged eye cornea 12 days after surgery;

          Fig. 4 shows fluorescein staining of damaged eye cornea following Intralipid™ treatment;

          Fig. 5 shows the histological appearance of damaged eye cornea following Lyteers™ treatment;

20           Fig. 6 shows fluorescein staining of damaged eye cornea after 3 days of treatment with HDL;

          Fig. 7 shows fluorescein staining of damaged eye cornea at the end of treatment with HDL;

25           Fig. 8 shows the histological appearance of damaged eye cornea after HDL treatment;

          Fig. 9 shows the histological appearance of damaged eye cornea following 7 days of treatment with Lipofundin™; and

          Fig. 10 shows the histological appearance of damaged eye cornea following 7 days of treatment with Lyteers™.

## DETAILED DESCRIPTION OF THE INVENTION

### I. Experimental Procedures

#### A. Animal model of corneal epithelium and conjunctival epithelium damage

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A rabbit model for keratoconjunctivitis (Gilbard *et al.*, *J. Inv. Ophthalm. Vis. Sci.*, 2:225-228 (1987)) was used with slight modification. Surgery performed on anesthetized rabbits using a surgical microscope (Inami, Japan) involved excision of the plical fold over the eye, occlusion of the lacrimal duct, and peeling of the palpebral and bulbar conjunctiva. This surgery was done on one eye of 20 rabbits of average age 3 months of both sexes. Surgery and all subsequent treatments were done in accordance with ARVO rules for animal care in research. Tear film osmolarity is elevated by postoperative day 1. Corneal epithelial glycogen levels decline progressively, and conjunctival goblet cell density decreases. These pathologies lead to corneal epithelium damage covering the entire corneal surface by the fifth postoperative day, and it was at this time that treatment of the eyes commenced.

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#### B. HDL preparation

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HDL was prepared from human plasma by differential ultracentrifugation flotation (Havel *et al.*, "Distribution and chemical composition of ultracentrifugally lipoproteins in human serum", *J. Clin. Invest.*, 34:1345-53. (1995)).

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#### C. Evaluation of Rabbit Cornea

Lesions in fluorescein stained corneas were clinically evaluated by biomicroscopy using a slit lamp (Haag Streit, Switzerland) with cobalt filter illumination. Photography of the fluorescein staining was taken with a slit lamp mounted camera (Topcon, Japan). At the end of each experiment, the rabbits were

sacrificed and the cornea were excised, fixed in paraformaldehyde and examined for epithelial lesions.

D. Histological examination

- 5                   At the end of the treatment, the rabbits were sacrificed by a lethal dose of intravenously injected pentobarbitone. The eyes were enucleated and the corneas fixed in 4% paraformaldehyde. Corneas were embedded in paraffin blocks, sectioned, and with hematoxylin-eosine for light microscope examination.

10    II.    **Treatment of corneal epithelium damage caused by conjunctival epithelium damage**

**Example 1**

- 15                   Rabbits with cornea damage induced as above were treated as follows: Five rabbits were treated with commercially available artificial tears (Lyteers™). five rabbits were treated with HDL (1mg protein/ml) in phosphate buffered saline. five rabbits were treated with a commercially available lipid mixture (10% Intralipid™: 10% soybean oil, 1.2% egg phospholipids, 2.2% glycerol), and two rabbits were left untreated. Treatment consisted of applying  
20   two drops to the eye 3 times a day for seven consecutive days. The eyes were evaluated clinically during the experiment and pictures were taken every other day of the fluorescein stained corneas.

- 25                   Fluorescein staining of damaged eyes 5 and 12 days following surgery is shown in Figs. 1 and 2, respectively. As can be seen, the surface of the untreated eye became progressively more scratched and opaque with time leading eventually to blurred vision.

                  Histological staining of damaged eye is shown in Fig. 3B and is compared to histological staining of normal eye 3A. As can be seen, in the damaged eye there has been complete erosion of the exposed epithelium due to

- 5     persisant rubbing of the epithelium by the lids as well as severe keratitis and vascularization of the cornea.

Animal eyes that were treated with Intralipid<sup>TM</sup> show complete reversal to normal morphological structure as indicated by fluorescein staining (Fig. 4). However, animal eyes treated with Lyeteers<sup>TM</sup>, (Fig. 5) (commercially available artificial tears composed mainly of water and a viscous substance) did not return to normal. In contrast to normal eyes, the damaged area did not become covered again with normal layers of cuboidal epithelial cells and a single top layer of wing cells, but was covered instead by only a single layer of wing cells. These results indicate that artificial tears such as Lyeteers<sup>TM</sup> cannot promote regeneration of normal eye epithelium.

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In contrast to this, animal eyes treated with HDL showed essentially a complete return to normal morphological structure as indicated by fluorescein staining taken on the third day (Fig. 6) and at the end of treatment (Fig. 7) as well as by histological staining (Fig. 8). Histological staining shows essentially a complete return to normal of the eye epithelium characterized by formation of several layers of cuboidal cells and a single top layer of wing cells.

These results clearly indicate that both HDL and Intralipid™ are able to promote healing and regeneration of damaged eye epithelium and return to normal epithelium.

**Example 2**

Three rabbits underwent central corneal peeling of the epithelium in both eyes. The area of peeling, 8 mm in diameter, was first demarcated with trephine, and the epithelium excised with a scalpel. The right eye of each rabbit was treated with two drops of Lipofundin™ 10% three times a day, while the left eye was treated with the same dose of Lyeteers™. The cornea were stained with fluorescein and photographed immediately after epithelium removal and on the third and fifth day afterwards. On the seventh day, the rabbits were sacrificed by a lethal dose of pentol and the corneas were excised, fixed and stained for light microscopy. The denuded area in the fluorescein and fixed corneas was determined, and the extent of remaining damage calculated as the remaining denuded area divided by the initial denuded area. The results appear in Table 1 below which gives the fraction of initial damage remaining 0, 3, 5, and 7 days after peeling the epithelium. The results show that the rate of healing was faster in the Lipofundin™ treated eyes. Figs. 9 and 10 show the epithelium of a Lipofundin™ treated eye and Lyeteers™ treated eye, respectively, after 7 days of treatment. Whereas the Lipofundin™ appears close to normal, in the Lyeteers™ treated eye the epithelium still appears thin, immature, and containing only flat and wing cells which have migrated to the denuded area.

Table 1

Time (Days)	Treatment	
	Lipofundin™	Lyeteers™
0	1.00	1.00
3	.304	.378
5	.036	.107
7	0	0

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